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Homo-Oligomerization of Marburgvirus VP35 Is Essential for Its Function in Replication and Transcription

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The nucleocapsid protein VP35 of Marburgvirus, a filovirus, acts as the cofactor of the viral polymerase and plays an essential role in transcription and replication of the viral RNA. VP35 forms complexes with the genome encapsidating protein NP and with the RNA-dependent RNA polymerase L. In addition, a trimeric complex had been detected in which VP35 bridges L and the nucleoprotein NP. It has been presumed that the trimeric complex represents the active polymerase bound to the nucleocapsid. Here we present evidence that a predicted coiled-coil domain between amino acids 70 and 120 of VP35 is essential and sufficient to mediate homo-oligomerization of the protein. Substitution of leucine residues 90 and 104 abolished (i) the probability to form coiled coils, (ii) homo-oligomerization, and (iii) the function of VP35 in viral RNA synthesis. Further, it was found that homo-oligomerization-negative mutants of VP35 could not bind to L. Thus, it is presumed that homo-oligomerization-negative mutants of VP35 are unable to recruit the polymerase to the NP/RNA template. In contrast, inability to homo-oligomerize did not abolish the recruitment of VP35 into inclusion bodies, which contain nucleocapsid-like structures formed by NP. Finally, transcriptionally inactive mutants of VP35 containing the functional homo-oligomerization domain displayed a dominant-negative phenotype. Inhibition of VP35 oligomerization might therefore represent a suitable target for antiviral intervention.

Marburgvirus (MARV) and the closely related Ebolavirus (EBOV) together make up the family *Filoviridae*, which is classified in the order *Mononegavirales*. MARV causes a fulminant hemorrhagic fever in humans and nonhuman primates with high fatality rates (28). To date, neither a vaccine nor a curative treatment for MARV infection in humans is available. However, live attenuated recombinant vaccines have been described which protected nonhuman primates against MARV and EBOV infections (23). These might represent promising candidate vaccines for human use as well. The recent outbreak of MARV disease in Angola underlines the emerging potential of this pathogen (4). The MARV particle is composed of seven structural proteins. Four of them, NP, VP35, VP30, and L, constitute the nucleocapsid complex of MARV, which surrounds the viral genome. NP, the major nucleocapsid protein, self-assembles into tubular nucleocapsid-like structures, which are found intracellularly in large inclusions (25, 29). The formation of the tubular structures by NP is presumed to represent the first step in the assembly of the nucleocapsid. NP interacts with VP35, which in turn interacts with the RNA-dependent RNA polymerase L (3). The complex of VP35 and L represents the active RNA-dependent RNA polymerase, with VP35 serving as a polymerase cofactor (33). Additionally, a trimeric complex was observed consisting of NP, VP35, and L, with VP35 connecting L and NP (3). Three of the four nucleocapsid proteins, NP, VP35, and L, are essential for transcription and replication of the viral RNA (32). The fourth nucleocapsid protein, VP30, plays an important role in viral transcription in the closely related EBOV. For MARV, the role of VP30 is not completely understood. While a minigenome-based transcription-replication system did not need VP30 for transcription (32), RNA interference-based downregulation of VP30 in MARV-infected cells simultaneously decreased the levels of all other viral proteins. This suggested an effect of VP30 on replication or transcription. When VP35 was downregulated by RNA interference, a significant reduction in the release of viral particles was recorded (12). Since VP35 is only very slightly phosphorylated, it differs from most other P proteins in the order *Mononegavirales* for the purpose of nomenclature. The term P protein has not been widely accepted for VP35 (2). However, VP35 is presumed to share most of the features that are displayed by P proteins of other viruses in the order *Mononegavirales*.

Although it is known that the P proteins of viruses of the rhabdo- and paramyxovirus families play a key role in transcription and replication, there is little knowledge about the actual function of these proteins at the molecular level. The P proteins are engaged in several protein complexes that might be important for the different presumed functions of the proteins. On the one hand, P proteins interact with N (or NP) that is organized in nucleocapsids (39, 44). On the other hand, P proteins also interact with the soluble N (or NP). This latter interaction is thought to keep N (or NP) in solution by a molecular chaperone activity of the P proteins (9). Additionally, P proteins are found in a complex with L, the catalytic subunit of the RNA-dependent RNA polymerase, and it was shown that the polymerase is only functional if the P protein is present (9, 10, 14, 15). Moreover, P proteins are part of a trimeric complex formed by NP, P, and L, which might reflect the active polymerase complex bound to the nucleocapsid or serve specifically as a replication complex (17). Finally, it has

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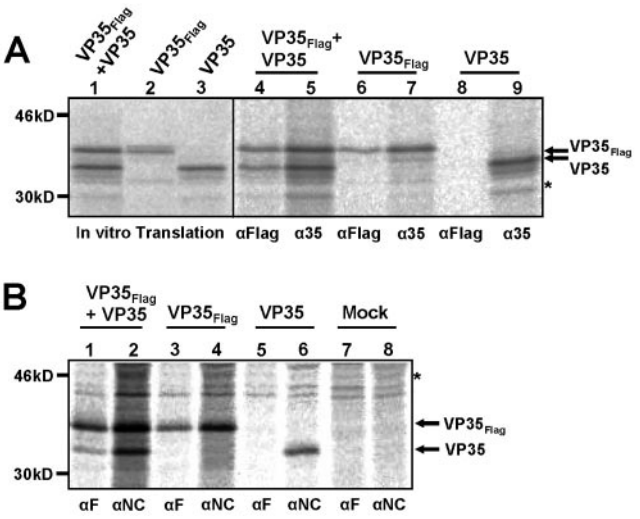


FIG. 1. Homo-oligomerization of $VP35_{Flag}$ and VP35. (A) $VP35_{Flag}$ and VP35 were coexpressed by using the TNT T7 Quick Coupled Reticulocyte Lysate System and metabolically labeled with [^{35}S]methionine. In vitro translation was followed by coimmunoprecipitation with an anti-Flag or an anti-VP35 antibody. One microliter of the translation reaction mixture and the immunocomplexes was separated by SDS-PAGE and visualized by Bio-Imager analysis. To exclude antibody cross-reactivities, $VP35_{Flag}$ and VP35 were singly expressed in vitro and precipitated with the indicated antibodies. The positions of the proteins are indicated. As a result of internal translation initiation in addition to the full-length products, smaller proteins are synthesized from the templates (*). (B) Huh-T7 cells were transfected with plasmids encoding $VP35_{Flag}$ (500 ng) and VP35 (500 ng) by using Lipofectamine Plus (Invitrogen) according the manufacturer's protocol. At 12 h posttransfection, cells were labeled with [^{35}S]Promix and the proteins were immunoprecipitated with an anti-Flag antibody or anti-NC serum directed against the nucleocapsid proteins NP, VP35, and VP30. Immunocomplexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. To exclude antibody cross-reactivities, $VP35_{Flag}$ and VP35 were singly expressed in vitro and precipitated with the indicated antibodies. *, unspecific cellular proteins.

been observed that P proteins can self-assemble via coiled coils (8, 18, 19).

In this study, we focused on homo-oligomerization of VP35 and found that the homo-oligomerization domain is located in the N terminus of the protein. Homo-oligomerization of VP35 is mediated by a coiled-coil motif, whose integrity is a prerequisite for the function of VP35 in replication and transcription since only the homo-oligomeric VP35 is able to interact with L, the catalytic subunit of the MARV polymerase. Transcriptionally inactive VP35 mutants that are homo-oligomerization competent display a dominant-negative effect. While formation of the MARV polymerase complex was strictly dependent on homo-oligomerization of VP35, the ability to bind to NP was still maintained in homo-oligomerization-negative VP35 mutants.

MATERIALS AND METHODS

Cells. Huh-T7 cells (kindly provided by V. Gauss-Müller, Department of Medical Molecular Biology, University of Lübeck, Lübeck, Germany) are derived from the human hepatoma cell line Huh7 and constitutively express the T7 RNA polymerase. Huh-T7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, L-glutamine, penicillin-streptomycin solution, and 1 mg/ml Geneticin (42). The cells were grown in an incubator at 37°C and 5% CO_2.

DNA plasmids and molecular cloning. The plasmid pTM1-VP35 contains the open reading frame of the VP35 gene of MARV (accession number Z12132) (40) under the control of the T7 RNA polymerase promoter (13, 32). The plasmid pTM1-$VP35_{Flag}$ was constructed by cloning the sequence encoding the Flag epitope to the 5′ end of the VP35 gene (11). Complementary oligonucleotides encoding the Flag epitope were hybridized in annealing buffer (70 mM Tris-HCl, pH 7.6; 7 mM $MgCl_2$; 10 mM dithiothreitol) by incubation for 1 min at 95°C and 20 min at 37°C, followed by incubation for 10 min at room temperature. Each oligonucleotide was used at a final concentration of 30 μM. The resulting double-stranded Flag epitope encoding DNA flanked by different restriction sites was purified by phenol-chloroform extraction and cloned into the digested pTM1-VP35 plasmid. The plasmid pTM1-L_{Flag}, which encodes the first 530 amino acids of MARV L, has been previously described (3).

Cloning of the VP35 deletion mutants. Detailed cloning strategies are available on request.

The plasmid pTM1-VP35 was used as the template for the construction of VP35 deletion mutants, which were verified by sequencing. The coding regions for the N-terminal and/or C-terminal deletion mutants of VP35 were amplified by PCR with proofreading *Pwo* polymerase (peqlab). The oligonucleotides used for PCR were supplied with different endonuclease restriction sites. For the N-terminal or C-terminal deletion mutants, a start codon or a stop codon was included in the oligonucleotides, respectively. After gel purification and digestion of the PCR fragments with the appropriate restriction endonucleases, the resulting DNA fragments were cloned into the vector pTM1.

Cloning of internal VP35 deletion mutants. To generate internal deletions in the VP35 gene, oligonucleotides in an inverted tail-to-tail orientation with a gap between their 5′ ends were designed. The gap encompassed the sequence to be deleted. The backbone of the vector and the target sequence was amplified by PCR with the proofreading *Pfu* Turbo DNA polymerase (Stratagene), and the resulting DNA was digested with DpnI and subsequently religated (22).

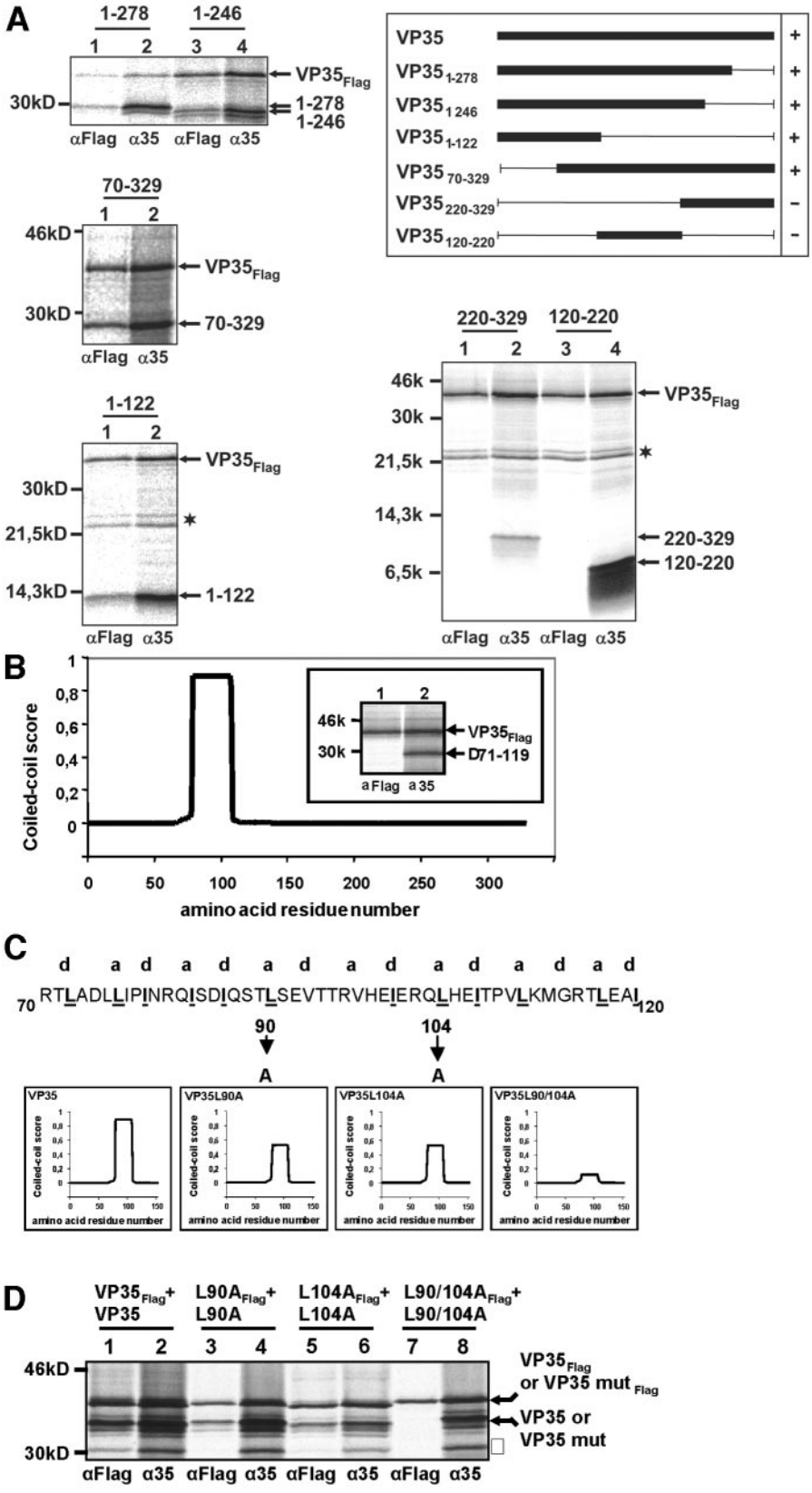
Cloning of VP35 substitution and chimeric mutants. Cloning of VP35 substitution mutants was performed with the QuikChange site-directed mutagenesis kit (Stratagene). The sequence encoding amino acids 70 to 120 of VP35 was amplified by PCR and fused to mutant $VP30_{2LA}$ of EBOV by insertion of the PCR fragment into the vector pT-$VP30_{2LA}$ or pT-$VP30_{2LAFlag}$ (20).

Computer analysis. For prediction of coiled-coil motifs, the amino acid sequence of VP35 was analyzed with the COILS 2.2 program (window 28) as described by Lupas et al. (26, 27).

In vitro translation and coimmunoprecipitation. The DNA plasmids described were used to in vitro translate $VP35_{Flag}$ or L_{Flag} simultaneously with VP35 or with VP35 mutants (final volume, 50 μl) and metabolically labeled with [^{35}S]methionine with the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the supplier's prescription. To achieve equal expression levels of the different Flag-tagged and untagged proteins, the respective DNA plasmids were used in optimized ratios. Afterwards, 5 μl of the in vitro translation reaction mixture was suspended in 500 μl Tris-KCl buffer (150 mM KCl; 10 mM Tris-HCl, pH 8.0; 0.1% NP-40, 3% bovine serum albumin) (38). Coimmunoprecipitation with the appropriate antibodies (anti-Flag monoclonal antibody M2 [Sigma] at a dilution of 1:500, guinea pig anti-VP35 serum at a dilution of 1:1,000, anti-VP30 rabbit immune serum at a dilution of 1:2,500, or anti-NC serum at a dilution of 1:500, as indicated in the legends to the figures) was performed as described elsewhere (30). Finally, the samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and analyzed by Bio-Imaging. To exclude antibody cross-reactivities, all VP35 mutants were singly expressed and precipitated with the indicated antibodies (data not shown).

DNA transfections and reporter gene assay. Huh-T7 cells (5×10^5 in a 7-cm^2 well) were transfected with plasmids encoding the MARV nucleocapsid proteins NP, VP35 (or/and VP35 mutants), and L and a MARV-specific minigenome, 3 M-5 M, containing the *cat* reporter gene, which is flanked by the leader and trailer regions of the MARV genome (32), by using FuGENE 6 (Roche) as described previously (31). Additionally, 0.5 μg of plasmid pC-T7Pol expressing the T7 RNA polymerase (kindly provided by T. Takimoto, St. Jude Children's Research Hospital, Memphis, Tenn., and Y. Kawaoka, University of Wisconsin, Madison, (34), were transfected. As a readout for transcription activity, chloramphenicol acetyltransferase (CAT) activity was determined by using a standard protocol (16). The radioactive signals were detected with a BAS-1000 Bio-Imaging Analyzer (Fujifilm) and TINA 2.0 software (Raytest).

RNA isolation and Northern blot analysis. Huh-T7 cells were transfected as described above. At 2 days posttransfection, the cells were lysed and cell lysates were treated with micrococcal nuclease (Fermentas). Subsequently, RNA isola-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tion was performed with the RNeasy kit (QIAGEN) as recommended by the manufacturer and subjected to Northern blot analysis as described by Mühlberger et al. (32).

Western blot analysis. Western blot analysis was carried out as described by Modrof et al. (30). The antibodies used and their respective dilutions are given in the figure legends.

Indirect immunofluorescence analysis. Huh-T7 cells were transfected with 0.1 μg pTM1-NP, 0.5 μg pTM1-$VP35_{Flag}$ (or Flag-tagged VP35 substitution mutants), or both plasmids by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Additionally, as described above, 0.5 μg of plasmid pC-T7Pol was cotransfected. The overall amount of transfected plasmids was held constant by adding empty vector (pTM1). Immunofluorescence analyses were performed as described previously (24).

RESULTS

VP35 forms homo-oligomers. Two of the nucleocapsid proteins of filoviruses have been shown to form homo-oligomers: MARV NP self-interacts to induce the formation of the helical nucleocapsid (25), and EBOV VP30 undergoes homo-oligomerization, which is necessary for the function of the protein during EBOV transcription (20). To investigate whether MARV VP35 has the ability to form homo-oligomers, we took advantage of the fact that VP35 and Flag-tagged VP35 ($VP35_{Flag}$) are distinguishable by their different migration velocities in SDS-PAGE (Fig. 1A, lanes 1 to 3). When $VP35_{Flag}$ was precipitated by using an anti-Flag antibody, the coexpressed VP35 was cosedimented (Fig. 1A, lane 4). Since the anti-Flag antibody did not recognize the untagged VP35 (Fig. 1A, lane 8), this result indicated that VP35 is able to homo-oligomerize. We coexpressed $VP35_{Flag}$ and VP35 in Huh-T7 cells and carried out coimmunoprecipitation with the cell lysates. Under these conditions, VP35 was found to homo-oligomerize as well (Fig. 1B, lane 1).

Mapping of the homo-oligomerization domain of MARV VP35. In a series of further coimmunoprecipitation analyses with a set of VP35 deletion mutants, we narrowed down the self-interacting site of VP35 to N-terminal amino acids 70 to 122 (Fig. 2A). In silico analyses revealed high prediction values for a coiled-coil motif between amino acids 70 and 120 (Fig. 2B). Since coiled-coil motifs are known to mediate protein-protein interactions (6, 27), it was presumed that the amino acids in this region are important for homo-oligomerization of VP35. To determine the function of the predicted coiled-coil motif, a deletion mutant of VP35 was constructed lacking amino acids 71 to 119 ($VP35_{\Delta 71\text{-}119}$). By coimmunoprecipitation analysis, it was shown that $VP35_{\Delta 71\text{-}119}$ was not able to interact with $VP35_{Flag}$ (Fig. 2B, insert). Therefore, a predicted coiled-coil domain between amino acids 70 and 120 is essential for homo-oligomerization of MARV VP35.

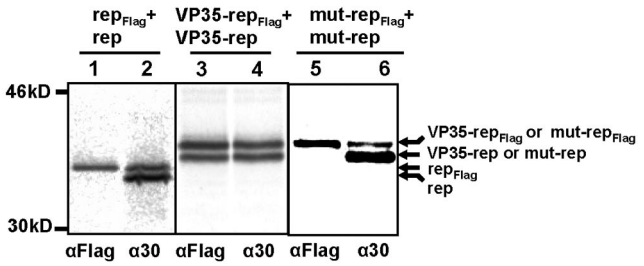


FIG. 3. The predicted coiled-coil domain of VP35 is sufficient to mediate homo-oligomerization. The putative homo-oligomerization domain spanning amino acids 70 to 120 was attached to a Flag-tagged (VP35-rep$_{Flag}$) and an untagged (VP35-rep) monomeric reporter protein. Leucine residues 90 and 104 within the homo-oligomerization domain of the generated constructs were replaced with alanine (mut-rep and mut-rep$_{Flag}$). The untagged monomeric reporter protein (rep) and the fusion proteins generated (VP35-rep, mut-rep) were coexpressed with their Flag-tagged versions, respectively. Complex formation was analyzed by coimmunoprecipitation with an anti-Flag monoclonal antibody and anti-VP30 rabbit immune serum. The proteins were separated by SDS-PAGE and visualized by Bio-Imager analysis. The positions of the proteins are indicated.

Next, we tested in silico the effect of leucine-to-alanine changes concerning the probability values for the formation of coiled coils. Replacing leucine 90 or leucine 104 diminished and replacing both residues abolished the probability to form coiled coils (Fig. 2C). Therefore, three VP35 mutants were constructed by replacing leucine 90 or 104 or both residues with alanine. Additionally, epitope-tagged versions of these three mutants were constructed with a Flag tag at the N terminus. The three mutants were, respectively, coexpressed pairwise (tagged and untagged) and subjected to coimmunoprecipitation analysis. Expression of all substitution mutants resulted in proteins with either slightly slower or faster electrophoretic mobility than wild-type VP35 (Fig. 2D). This was most likely due to changes in SDS binding. Figure 2D shows that while single leucine-to-alanine changes only marginally influenced the homo-oligomerization ability of VP35 (Fig. 2D, lanes 3 to 6), replacing both leucines abolished homo-oli-

FIG. 2. Localization of the homo-oligomerization domain with VP35 mutants. (A) Coimmunoprecipitation of $VP35_{Flag}$ and VP35 mutants. Flag-tagged VP35 was cotranslated with VP35 mutants with the TNT T7 Quick Coupled Reticulocyte Lysate System and metabolically labeled with [^{35}S]methionine. In vitro translation was followed by coimmunoprecipitation with an anti-Flag or an anti-VP35 antibody. Immunocomplexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. The positions of Flag-tagged VP35 and VP35 mutants are indicated. As a result of internal translation initiation in addition to the full-length product, smaller proteins were synthesized from the templates (*). A schematic representation of the MARV VP35 deletion mutants used for coimmunoprecipitation analyses with $VP35_{Flag}$ is shown at the upper right. The subscript numbers refer to the amino acids of VP35 in the respective mutants. A plus sign indicates an interaction with $VP35_{Flag}$; a minus sign indicates no interaction with $VP35_{Flag}$. (B) In silico analysis of the amino acid sequence of VP35 with the COILS 2.2 program. The graph shows a high probability of a coiled-coil structure between amino acids 70 and 120. (Insert) $VP35_{Flag}$ and the VP35 mutant lacking the potential coiled-coil domain ($VP35_{\Delta 71\text{-}119}$) were cotranslated and analyzed for interaction by coimmunoprecipitation with a Flag-specific and an anti-VP35 antibody. (C) Amino acid sequence of the presumed coiled-coil domain. Note the occurrence of hydrophobic amino acid residues at the first (a) and fourth (d) positions of the heptad repeat (bold and underlined). Results of an in silico analysis of the VP35 substitution mutants (leucine 90 and/or 104 changed to alanine) with the COILS 2.2 program is shown at the bottom. (D) Flag-tagged VP35 substitution mutants were cotranslated with untagged VP35 substitution mutants and analyzed for interaction by coimmunoprecipitation as described above. The positions of the proteins are shown. As a result of internal translation initiation in addition to the full-length products, smaller proteins were synthesized from the templates (*).

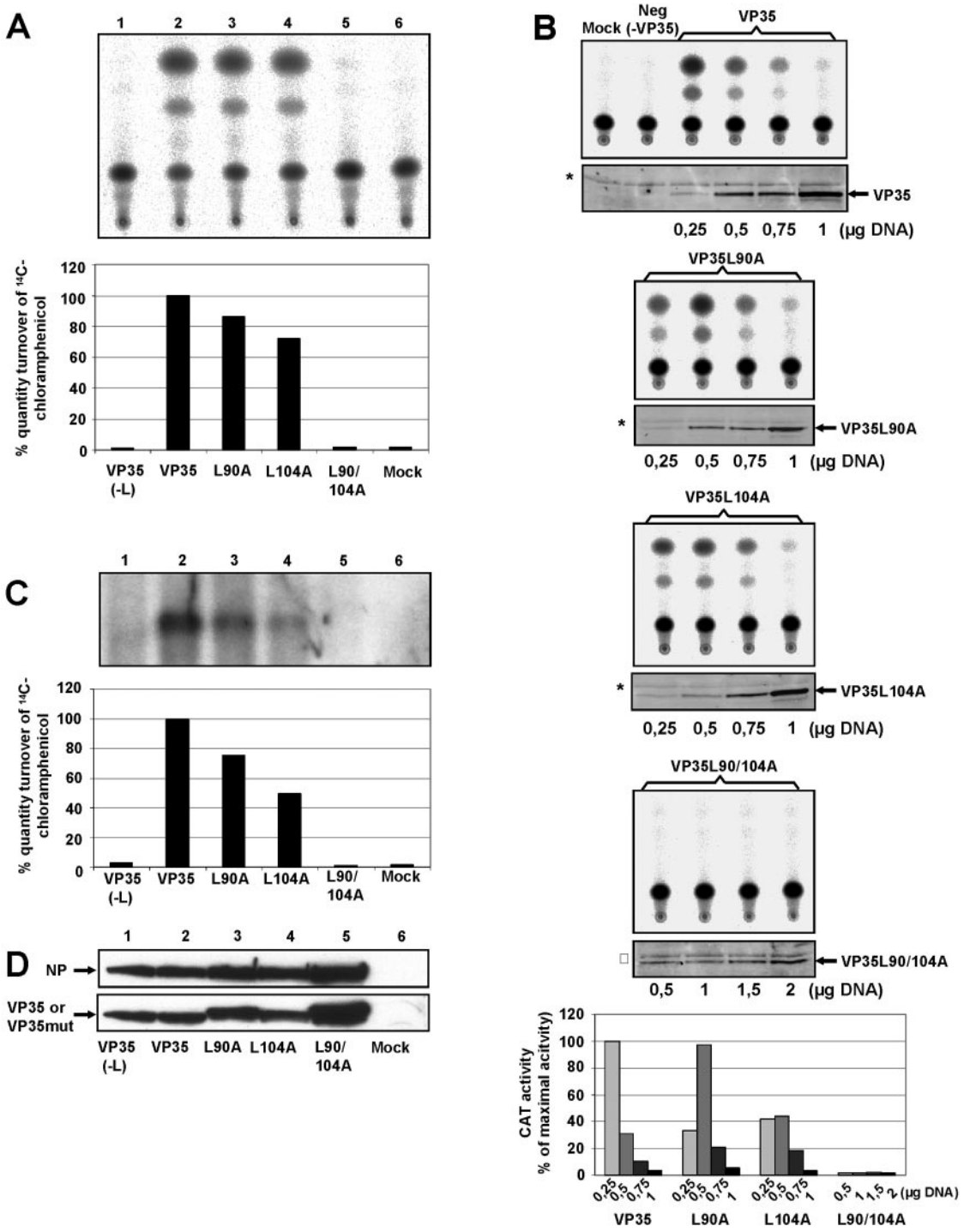


FIG. 4. Influence of homo-oligomerization of VP35 on MARV transcription and replication. (A) Transcription analysis. Huh-T7 cells (5×10^5) were transfected with plasmids encoding the MARV nucleocapsid proteins NP, L, and VP35 and VP35 substitution mutants. Additionally, a plasmid encoding the MARV-specific artificial minigenome 3 M-5 M and a plasmid expressing the T7 RNA polymerase were transfected. At 2 days posttransfection, cells were lysed and CAT activity was determined with [^{14}C]chloramphenicol and acetyl coenzyme A as a substrate. The acetylated chloramphenicol was separated via thin-layer chromatography. Since chloramphenicol has two acetylation sites and the respective products have different migration velocities in thin-layer chromatography, two products can be distinguished beside the slow-migrating nonacetylated chloramphenicol when CAT activity is present. Quantification of CAT activity was done by the TINA 2.0 software (Raytest). (B) Titration of VP35 and VP35 substitution mutants. Huh-T7 cells were transfected as described above. The transfected amounts of VP35 or VP35 substitution mutants are indicated. Quantification of CAT activity was done by the TINA 2.0 software (Raytest). As the primary antibody for Western blotting, guinea pig anti-VP35 serum (dilution, 1:5,000) was used, and as the secondary antibody, a peroxidase-coupled anti-guinea pig antibody (DAKO; dilution, 1:25,000) was used. *, unspecific cellular proteins. Quantitative presentation of the titration of VP35, VP35L90A, and VP35L104A. (C) Replication

gomerization completely (Fig. 2D, lanes 7 and 8). This result showed that the interaction of VP35 with itself depended strongly on the formation of a coiled-coil structure in the N terminus of the protein.

The coiled-coil motif is sufficient for mediating protein-protein interaction. It was then investigated whether the coiled-coil motif detected was sufficient to mediate the interaction between VP35 molecules. For this, the coiled-coil motif was fused to a monomeric reporter protein. As a reporter we chose a mutant of EBOV VP30 ($VP30_{2LA}$) that has recently been shown to be inefficient in the formation of homo-oligomers (20). The sequence encoding the coiled-coil motif of VP35 was cloned 5′ to the open reading frame of $VP30_{2LA}$. Additionally, a Flag-tagged fusion protein was constructed and both proteins were coexpressed in vitro. As a control, we coexpressed the tagged and untagged versions of the reporter protein and subjected all samples to coimmunoprecipitation analysis. While the reporter protein did not interact with itself (Fig. 3, lane 1), the fusion protein with the N-terminal coiled-coil domain was homo-oligomerization competent (Fig. 3, lane 3). A fusion protein in which leucine residues 90 and 104 were replaced with alanine was not able to support interaction of the monomeric reporter protein (Fig. 3, lane 5). It was therefore concluded that the coiled-coil domain in the N terminus of VP35 was able to mediate $VP30_{2LA}$ homo-oligomerization.

Function of homo-oligomerization of VP35 for transcription and replication. Next, we analyzed the function of homo-oligomerization of VP35 for transcription and replication of viral RNA by using a MARV-specific minigenome system (32). The MARV-specific minigenome contained all necessary *cis*-active RNA elements and a *cat* reporter gene. The minigenome-containing plasmid was transfected into Huh-T7 cells together with plasmids encoding the nucleocapsid proteins NP, L, and VP35. These three proteins have been shown earlier to be essential and sufficient for MARV-specific transcription and replication. Transcription of the minigenome by the viral proteins results in reporter gene expression, which is monitored by CAT activity (32). With this system, we replaced VP35 with the VP35 mutants containing leucine-to-alanine changes (Fig. 2C). We found that replacing single leucine residues in the coiled-coil motif of VP35 resulted in mutants that were still able to support MARV-specific transcription, although less efficiently than wild-type VP35 (Fig. 4A, lanes 3 and 4). Replacement of two leucine residues, however, which abolished the self-interaction of VP35, abolished the function of VP35 completely (Fig. 4A, lane 5). Titration experiments with the VP35 substitution mutants supported these results. Mutant $VP35_{L90A}$ was slightly less effective in supporting transcription activity than wild-type VP35. $VP35_{L104A}$ was even more impaired in its function, while even very high concentrations of the double mutant $VP35_{L90/104A}$ did not support polymerase activity (Fig. 4B). The effect of leucine-to-alanine mutations on the replica-

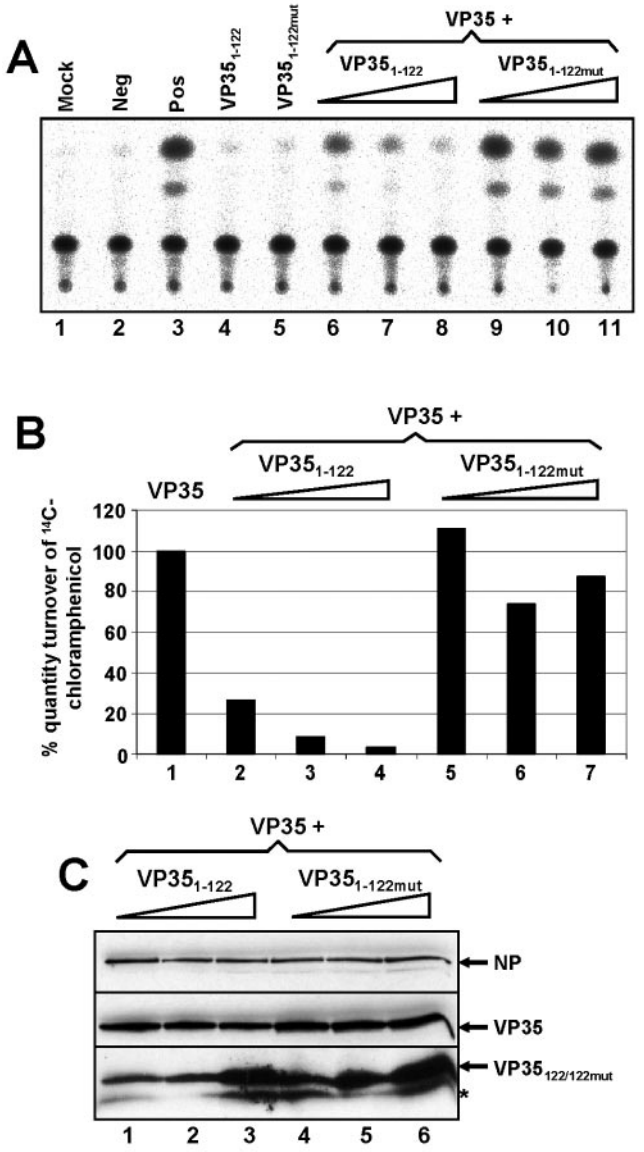


FIG. 5. Impact of mixed oligomers derived from VP35 and VP35 truncation mutants on MARV transcription. (A) The minigenome system was set up in 5×10^5 Huh-T7 cells as described in the legend to Fig. 4A. VP35 was replaced either with $VP35_{1-122}$ or $VP35_{1-122mut}$ or with a mixture of VP35 (500 ng) and either $VP35_{1-122}$ or $VP35_{1-122mut}$ in increasing amounts. A CAT assay was performed, and the radioactive signals were detected with a Bio-Imaging Analyzer. (B) Quantification of CAT activity was done by the TINA 2.0 software (Raytest). (C) Western blot assay. The expression levels of NP, VP35, and the respective mutants were checked by Western blot analysis. The antibodies used for detection of NP, VP35, and VP35 mutants are described in the legend to Fig. 4C.

analysis. Huh-T7 cells were transfected as described above. At 2 days posttransfection, cell lysates were treated with micrococcal nuclease. The isolated RNA was used to perform Northern blot analysis with digoxigenin-labeled riboprobes. Quantification of CAT activity was done by the TINA 2.0 software (Raytest). (D) Western blot assay. NP was detected by a mouse monoclonal anti-NP antibody (dilution, 1:1,000) and a peroxidase-coupled anti-mouse antibody (DAKO; dilution, 1:40,000). The expression levels of the respective mutants were checked by Western blot analysis. As the primary antibody, a guinea pig anti-VP35 serum (dilution, 1:5,000) was used, and as secondary antibody, a peroxidase-coupled anti-guinea pig antibody (DAKO; dilution, 1:25,000) was used.

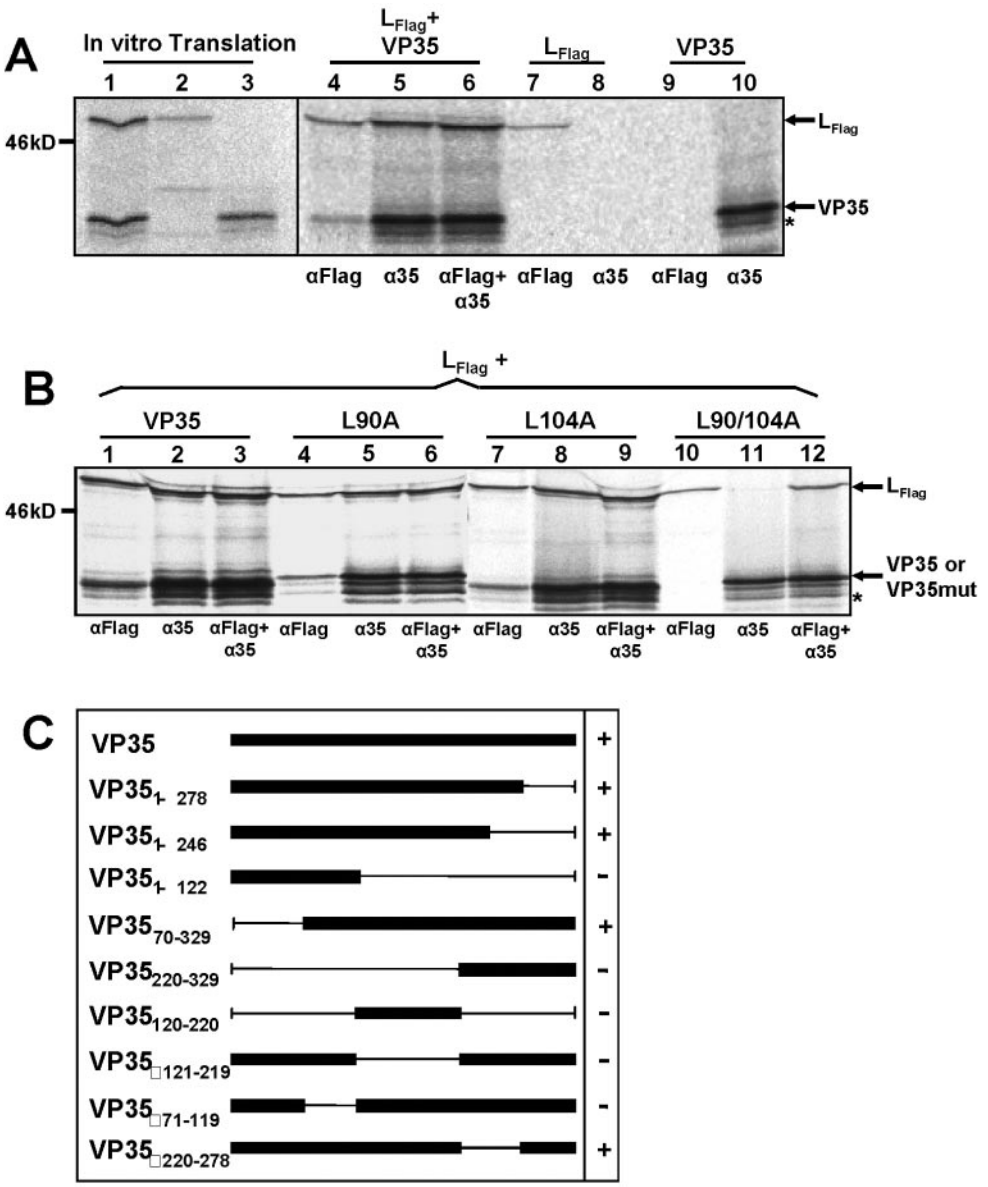


FIG. 6. Influence of homo-oligomerization of VP35 on interaction with L. (A) L_{Flag} and VP35 were coexpressed with the TNT T7 Quick Coupled Reticulocyte Lysate System and metabolically labeled with [^{35}S]methionine. In vitro translation was followed by coimmunoprecipitation with an anti-Flag and/or an anti-VP35 antibody. Immunocomplexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. To exclude antibody cross-reactivities, L_{Flag} and VP35 were singly expressed in vitro and precipitated with the indicated antibodies. The positions of the proteins are indicated. (*) In addition to the full-length products, smaller proteins are synthesized from the templates as a result of internal translation initiation. (B) The untagged VP35 substitution mutants (leucine 90 and/or 104 changed to alanine) were cotranslated with L_{Flag} and analyzed for interaction by coimmunoprecipitation as described above. The positions of the proteins are shown. (*) In addition to the full-length product, smaller proteins are synthesized from the templates as a result of internal translation initiation. (C) Schematic representation of the MARV VP35 deletion mutants used for coimmunoprecipitation analyses with L_{Flag}. The subscript numbers refer to the amino acids of VP35 in the respective mutants. A plus sign indicates an interaction with L_{Flag}; a minus sign indicates no interaction with L_{Flag}.

tion of the minigenome was also tested. Replicated minigenome RNA, in contrast to transcribed minigenome RNA, is resistant against RNase digestion because it is encapsidated by the major nucleocapsid protein NP (32). A Northern blot analysis was carried out with RNA purified from Huh-T7 cells expressing the nucleocapsid proteins of MARV and the MARV-specific minigenome that, prior to purification, was digested with nuclease. It was possible to detect nuclease-resistant, and thus replicated, RNA in samples that contained either VP35 or the single leucine-to-alanine VP35 mutants (Fig. 4C, lanes 3 and 4), although the amount of replicated RNA was decreased when the single leucine-to-alanine mutants were employed. When VP35 was replaced with the double leucine-to-alanine mutant, no replicated RNA was detected (Fig. 4C, lane 5). To make sure that similar amounts of VP35 and mutants of VP35 were present in the samples, ex-

pression was checked by Western blot analysis (Fig. 4D). The data obtained suggest that homo-oligomerization of VP35 is a prerequisite for the function of the protein in viral RNA transcription and replication. Moreover, the reduction in the activity of single leucine-to-alanine mutants correlated with the decreased ability to form coiled-coil structures.

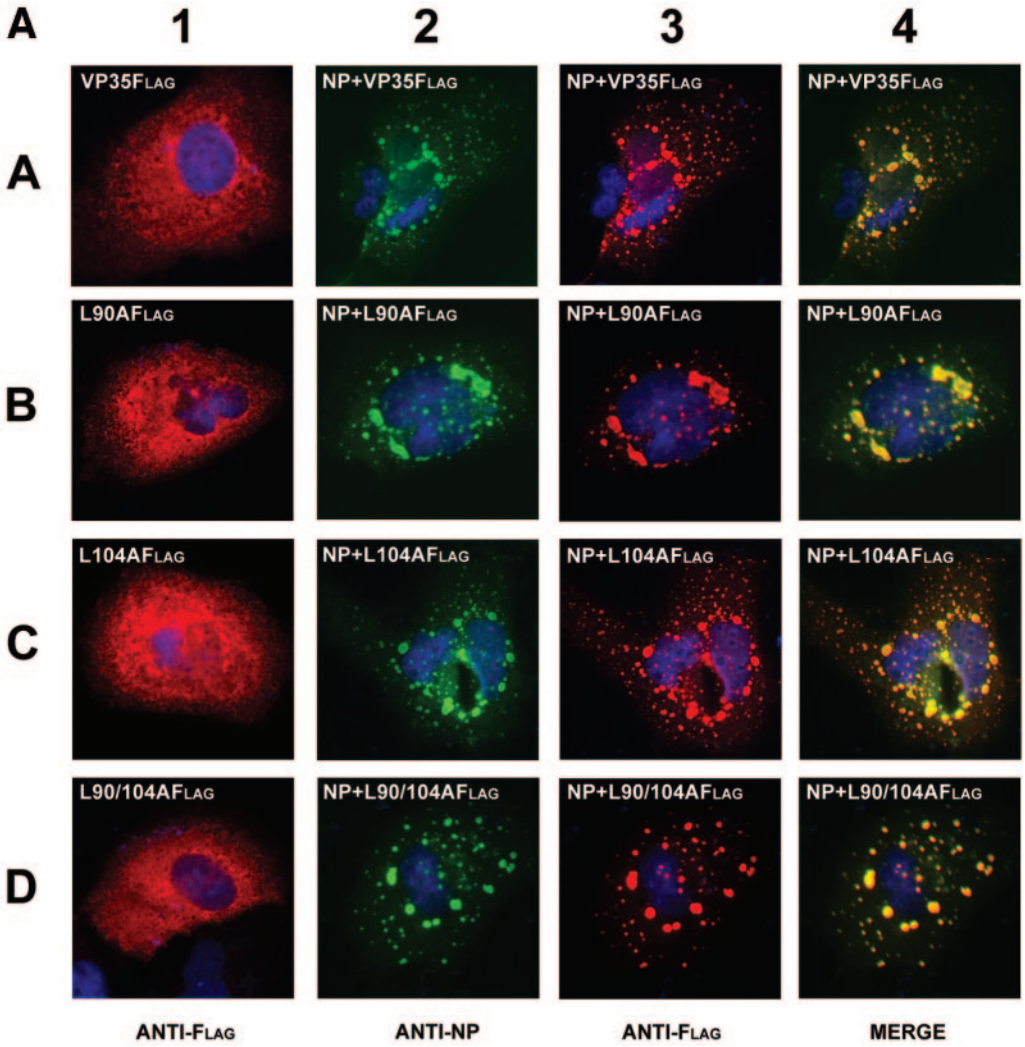
Homo-oligomerization-competent deletion mutants of VP35 are dominant negative. This led to the hypothesis that if homo-oligomerization of VP35 is essential for the protein's function, mutants of VP35 that are homo-oligomerization competent but functionally negative should display a dominant-negative phenotype. To test this hypothesis, we replaced VP35 in the MARV-specific minigenome system with either an N-terminal deletion mutant containing an intact homo-oligomerization motif ($VP35_{1\text{-}122}$) or the same N-terminal deletion mutant with a destroyed homo-oligomerization motif (L90/104A), which was named $VP35_{1\text{-}122mut}$. As expected, replacing VP35 with either of the mutants did not result in a functional transcription system (Fig. 5, lanes 4 and 5). Next, the minigenome system was set up by replacing VP35 with a mixture of VP35 and different concentrations of the respective deletion mutants. The result is shown in Fig. 5, lanes 6 to 11. The transcriptional activity of the system was diminished in a concentration-dependent manner in the presence of VP35 and the homo-oligomerization-competent mutant ($VP35_{1\text{-}122}$; Fig. 5A, lanes 6 to 8). Samples that contained the homo-oligomerization-incompetent mutant ($VP35_{1\text{-}122mut}$) displayed CAT activity that was close to normal levels (Fig. 5A, lanes 9 to 11). Quantitative analysis showed that the greatest amount of $VP35_{122}$ reduced CAT activity to 5% of the control level (Fig. 5B). Since it is known that the ratio of NP to VP35 is critical for this system, we checked whether the levels of NP and VP35 were changed by the coexpression of the mutants of VP35 (32). Western blot analysis of Huh-T7 cell lysates showed that the levels of NP and VP35 remained constant and only the levels of the deletion mutants were increased with increasing amounts of transfected DNA plasmids (Fig. 5C). It is concluded that, in accord with the hypothesis, homo-oligomerization-competent but transcriptionally inactive deletion mutants of VP35 display a dominant-negative phenotype. This result supports the idea that intact homo-oligomers of VP35 are necessary for the function of the protein during the transcription and replication of viral RNA.

Monomeric VP35 is inhibited in its interaction with polymerase L. VP35 interacts with L to form the active viral RNA-dependent RNA polymerase. The binding site of VP35 on L is located within the first 530 amino acids (3). For the investigation of the interaction between L and VP35, we used a Flag-tagged mutant of L (L_{Flag}) spanning the first 530 amino acids (3). VP35 and L_{Flag} were cotranslated in vitro and immunoprecipitated with monospecific antibodies. In Fig. 6A, lanes 4 to 6, it is shown that, as expected, the anti-Flag antibody precipitated not only L_{Flag} but also VP35. Vice versa, the anti-VP35 antibody precipitated L_{Flag} together with VP35. To exclude antibody cross-reactivities, L_{Flag} and VP35 were singly expressed and precipitated with the indicated antibodies (Fig. 6A, lanes 7 to 10). L_{Flag} was not precipitated with the anti-VP35 antibody, nor was VP35 precipitated with the anti-Flag antibody. We next investigated whether the binding site of L_{Flag} on VP35 overlaps the VP35 homo-oligomerization site. By performing coimmunoprecipitation analyses of L_{Flag} coexpressed with deletion mutants of VP35, we obtained the following results (Fig. 6C). Removal of the C-terminal amino acids of VP35 up to position 246 did not result in a significant diminution of the strength of binding between L_{Flag} and VP35 mutants. Further truncation of VP35 until amino acid 122, however, abolished the binding to L_{Flag} completely ($VP35_{1\text{-}122}$). The importance of the region between amino acids 120 and 220 for binding to L_{Flag} was underlined by the finding that the internal deletion of these amino acids also inhibited binding ($VP35_{\Delta 121\text{-}219}$). However, a construct consisting only of amino acids 120 to 220 was not sufficient to mediate the interaction with L_{Flag} ($VP35_{120\text{-}220}$). Additionally, a construct missing amino acids 71 to 119 failed to bind to L_{Flag} ($VP35_{\Delta 71\text{-}119}$). In conclusion, an essential interaction domain for the binding of L_{Flag} on VP35 was located between amino acids 121 and 219. Moreover, the domain that had been shown to contain the VP35 homo-oligomerization domain (70 to 120) was also necessary for binding to L_{Flag}.

Next, we were interested in determining whether VP35 mutants that were not able to homo-oligomerize could still interact with L_{Flag}. We therefore employed the leucine-to-alanine mutants ($VP35_{L90A}$, $VP35_{L104A}$, and $VP35_{L90/104A}$) in a coimmunoprecipitation assay with L_{Flag}. While the single-leucine mutants still were able to interact with L_{Flag} (Fig. 6B, lanes 4 to 9), the homo-oligomerization-negative double mutant was not (Fig. 6B, lanes 10 to 12). Thus, homo-oligomerization of VP35 is a prerequisite for binding to L.

Monomeric VP35 interacts with the nucleoprotein NP. It was shown previously that MARV VP35 interacts with the major nucleocapsid protein NP (3). NP is the main determinant of the nucleocapsid complex and forms, upon recombinant expression, intracellular inclusions. These inclusions contain accumulated tubular structures, which correspond to newly formed nucleocapsids inside viral inclusions of MARV-infected cells (25). When coexpressed with NP, the otherwise homogeneously distributed $VP35_{Flag}$ was recruited into the NP-induced inclusions (Fig. 7, top, A1 to A4). It was next of interest to determine whether homo-oligomerization of VP35 is essential for the ability of the protein to interact with NP. The distribution of the leucine-to-alanine mutants ($VP35_{L90AFlag}$, $VP35_{L104AFlag}$, and $VP35_{L90/104AFlag}$) was analyzed by immunofluorescence analysis. Upon solitary expression, the VP35 mutants were homogeneously distributed in the cytoplasm of the transfected cells (Fig. 7, top, B1 to D1). Upon coexpression with NP, all of the VP35 mutants were colocalized with the NP inclusions (Fig. 7, top, B2 to B4, C2 to C4, and D2 to D4). Additionally, we performed experiments in which NP was coimmunoprecipitated with $VP35_{Flag}$ or the oligomerization-negative mutant $VP35_{L90/104AFlag}$ after coexpression in Huh-T7 cells. We found that, in accordance with the immunofluorescence analyses, $VP35_{Flag}$ and the double mutant $VP35_{L90/104AFlag}$ did coprecipitate with NP (Fig. 7, bottom, lanes 1 and 7). It was therefore concluded that homo-oligomerization of VP35 is not essential for the interaction between VP35 and NP.

Together, the data presented showed that an N-terminal coiled-coil motif in VP35 is essential and sufficient for homo-oligomerization of the protein. While homo-oligomerization is



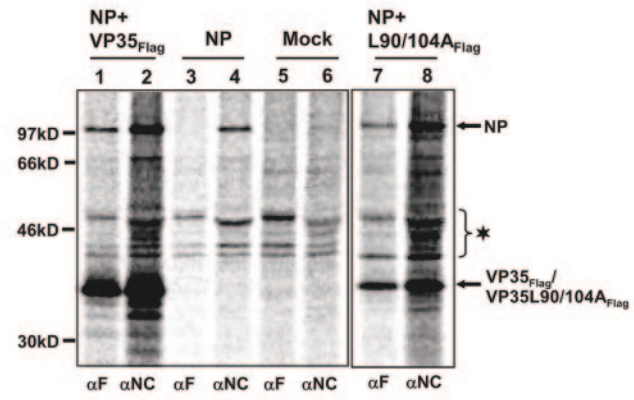


FIG. 7. Influence of the homo-oligomerization of VP35 on colocalization of VP35 and NP-induced inclusions. (Top) $VP35_{Flag}$ or the Flag-tagged VP35 substitution mutants were either singly expressed or coexpressed together with NP. Immunofluorescence analysis was performed with a rabbit anti-Flag antibody (Sigma; dilution, 1:200) and a mouse monoclonal anti-NP antibody (dilution, 1:25). As secondary antibodies, a goat anti-rabbit immunoglobulin G antibody conjugated with rhodamine and a goat anti-mouse antibody conjugated with fluorescein isothiocyanate (Dianova, dilution, 1:200) were used. $VP35_{Flag}$ was expressed singly (A1) or coexpressed with NP (A2 to A4). (B to D) Single expression of the VP35 substitution mutant $L90A_{Flag}$, $L104A_{Flag}$, or $L90/104A_{Flag}$ (B1 to D1) versus coexpression together with NP (B2 to B4, C2 to C4, D2 to D4). (Bottom) Huh-T7 cells were transfected with plasmids encoding NP (100 ng) and $VP35_{Flag}$ or VP35 substitution mutant $L90/104A_{Flag}$ (500 ng) with Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. To exclude antibody cross-reactivities, NP was singly expressed and precipitated with the indicated antibodies. At 12 h posttransfection, cells were labeled with [^{35}S]Promix and the proteins were immunoprecipitated with an anti-Flag antibody or an anti-NC serum, which is directed against the nucleocapsid proteins NP, VP35, and VP30. Immunocomplexes were separated by SDS-PAGE and visualized by Bio-Imager analysis. *, unspecific cellular proteins.

[1]dispensable for the recruitment of VP35 into the NP-induced inclusions, it is essential for the function of the protein in replication and transcription since monomeric VP35 is not able to recruit the polymerase to the NP/RNA template.

DISCUSSION

In the present study, we have mapped the homo-oligomerization domain of VP35 to amino acids 70 to 120. This region contains a predicted coiled-coil motif whose integrity is crucial for homo-oligomerization of VP35. The mutation of only two amino acids within the predicted coiled-coil motif renders VP35 incapable to homo-oligomerization. Amino acids 70 to 120 were also sufficient to confer interaction of an otherwise monomeric reporter protein.

Coiled-coil motifs are frequently used to join P monomers in members of the order *Mononegavirales*. A high prediction value for the formation of a coiled-coil motif is found not only in the N terminus of MARV VP35 but also in the N terminus of the closely related EBOV VP35. It is presumed that homo-oligomerization of EBOV VP35, like MARV VP35, is dependent on this motif for homo-oligomerization. Moreover, most of the presumed homo-oligomerization domains of analogous P proteins of viruses in the order *Mononegavirales* contain coiled-coil motifs as well (8). These motifs are located in different parts of the respective P proteins. In P proteins of the family *Paramyxoviridae*, the coiled-coil motif is mostly located in the C terminus (1, 5, 8, 10, 21, 35, 43, 45, 46). For the vesiculoviruses, it was found that N-terminal parts of the P proteins with an elevated coiled-coil potential are involved in homo-oligomerization (8, 36). Thus, with few exceptions (8, 15), coiled-coil motifs are presumed to serve as interaction domains for the homo-oligomerization of the P proteins of viruses in the order *Mononegavirales*. This is an interesting observation since the overall sequence homology between the P proteins of members of the order *Mononegavirales* is low and the coiled-coil motifs are located at different places in the primary sequence of the different P proteins. The module responsible for the homo-oligomerization of the P proteins is therefore functionally independent of its localization within the respective protein. This assumption is consistent with our finding that the coiled-coil motif of VP35 confers protein-protein interaction even if it is placed at the very N terminus of a reporter protein. Similar to this, Curran et al. showed that the coiled-coil motif of measles virus was able to induce homo-oligomerization of the otherwise monomeric La protein (8). Taken together, our study confirms and extends previous data showing that coiled-coil motifs are peptide modules that are used throughout the order *Mononegavirales* to confer homo-oligomerization of the polymerase cofactor P.

Homo-oligomerization is essential for the function of VP35 in replication and transcription but not for the interaction with NP-induced inclusions. Homo-oligomerization of VP35 was found to be essential for the function of the protein during transcription and replication. Most likely, the inability of monomeric VP35 to support transcription and replication was due to an impaired interaction with polymerase L. Since monomeric VP35 cannot serve as a bridge between the NP/RNA template and L, it is conceivable that L cannot be recruited to the template and therefore transcription and replication cannot take place. Similar data have been published for vesicular stomatitis virus (VSV). Only homo-oligomeric VSV P is able to bind to the catalytic polymerase subunit L, and monomeric VSV P is unable to support viral transcription and replication (14). A different situation is reported for the rinderpest virus (family *Paramyxoviridae*). Ramahan and colleagues showed that although the homo-oligomerization-incompetent P protein did not support transcription, it still bound L (37). An intermediate phenotype has been found with human parainfluenza virus type 3. In comparison with wild-type P, inactive monomeric P is impaired but not blocked in its ability to bind L (5). For the fourth family within the order *Mononegavirales*, *Bornaviridae*, it has also been shown recently that homo-oligomerization of the P protein is essential for the function of the protein in transcription. However, as with rinderpest virus, monomeric P is bound to L (41). This finding is in agreement with the presumption that the homo-oligomerization of P proteins of viruses in the order *Mononegavirales* is critically important to establish the functional state of the polymerase complex. As a working hypothesis to explain the need for homo-oligomeric P proteins, it has been proposed that the different monomers are sequentially bound and released from the N/RNA template, thereby cartwheeling the polymerase complex along the N/RNA template (7). In the presence of only monomeric P, this locomotion would not be possible. Our study highlights another facet showing that already formation of the functional polymerase needs VP35 in a conformation that is obviously only displayed by the homo-oligomer. Since the polymerase complex is recruited to the template by VP35, it is evident that monomeric VP35 cannot support transcription and replication. Experiments to reconstitute the function of the polymerase complex by insertion of an irrelevant coiled-coil domain into the oligomerization-negative VP35 mutant, which would additionally demonstrate the essential role of the coiled-coil-mediated homo-oligomerization, are under way.

The constructed homo-oligomerization-negative VP35 mutants were still able to interact with NP in inclusions that contain newly formed nucleocapsid-like structures. This result points, on the one hand, to the fact that the inserted mutations did not lead to a complete loss of function of VP35. On the other hand, this observation further supports the idea that oligomerization of nucleocapsid proteins is important to trigger different functions of the protein.

Finally, we were able to show that negative mutants of VP35, which were still able to homo-oligomerize, displayed a dominant-negative phenotype in transcription and replication of viral RNA. This result suggests that only complexes composed of functional VP35 proteins are able to support transcription and replication. Further support for this idea comes from results published by Rahaman et al. showing that homo-oligomerization-competent but functionally negative mutants of the rinderpest virus P protein display a dominant-negative phenotype (37). Possibly, this feature can be addressed in the quest to develop antivirals against MARV.

In summary, we have shown that the integrity of a coiled-coil structure in the N terminus is essential and sufficient for homo-oligomerization of VP35. Homo-oligomerization of VP35, in turn, is necessary for the transcription and replication of viral RNA since monomeric VP35 is not able to recruit L to the NP/RNA template.

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REFERENCES

1. **Asenjo, A., and N. Villanueva.** 2000. Regulated but not constitutive human respiratory syncytial virus (HRSV) P protein phosphorylation is essential for oligomerization. FEBS Lett. **467:**279–284.
2. **Becker, S., and E. Mühlberger.** 1999. Co- and posttranslational modifications and functions of Marburg virus proteins. Curr. Top. Microbiol. Immunol. **235:**23–34.
3. **Becker, S., C. Rinne, U. Hofsäss, H.-D. Klenk, and E. Mühlberger.** 1998. Interactions of Marburg virus nucleocapsid proteins. Virology **249:**406–417.
4. **Centers for Disease Control and Prevention.** 2005. Outbreak of Marburg virus hemorrhagic fever—Angola, October 1, 2004-March 29, 2005. Morb. Mortal. Wkly. Rep. **54:**308–309.
5. **Choudhary, S. K., A. G. Malur, Y. Huo, B. P. De, and A. K. Banerjee.** 2002. Characterization of the oligomerization domain of the phosphoprotein of human parainfluenza virus type 3. Virology **302:**373–382.
6. **Cohen, C., and D. A. Parry.** 1994. Alpha-helical coiled coils: more facts and better predictions. Science **263:**488–489.
7. **Curran, J.** 1998. A role for the Sendai virus P protein trimer in RNA synthesis. J. Virol. **72:**4274–4280.
8. **Curran, J., R. Boeck, N. Lin-Marq, A. Lupas, and D. Kolakofsky.** 1995. Paramyxovirus phosphoproteins form homotrimers as determined by an epitope dilution assay, via predicted coiled coils. Virology **214:**139–149.
9. **Curran, J., J. B. Marq, and D. Kolakofsky.** 1995. An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J. Virol. **69:**849–855.
10. **De, B. P., M. A. Hoffman, S. Choudhary, C. C. Huntley, and A. K. Banerjee.** 2000. Role of NH_2- and CO-OH-terminal domains of the P protein of human parainfluenza virus type 3 in transcription and replication. J. Virol. **74:**5886–5895.
11. **Einhauer, A., and A. Jungbauer.** 2001. The FLAG peptide, a versatile fusion tag for the purification of recombinant proteins. J. Biochem. Biophys. Methods **49:**455–465.
12. **Fowler, T., S. Bamberg, P. Möller, H. D. Klenk, T. F. Meyer, S. Becker, and T. Rudel.** 2005. Inhibition of Marburg virus protein expression and viral release by RNA interference. J. Gen. Virol. **86:**1181–1188.
13. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
14. **Gao, Y., N. J. Greenfield, D. Z. Cleverley, and J. Lenard.** 1996. The transcriptional form of the phosphoprotein of vesicular stomatitis virus is a trimer: structure and stability. Biochemistry **35:**14569–14573.
15. **Gigant, B., F. Iseni, Y. Gaudin, M. Knossow, and D. Blondel.** 2000. Neither phosphorylation nor the amino-terminal part of rabies virus phosphoprotein is required for its oligomerization. J. Gen. Virol. **81:**1757–1761.
16. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
17. **Gupta, A. K., D. Shaji, and A. K. Banerjee.** 2003. Identification of a novel tripartite complex involved in replication of vesicular stomatitis virus genome RNA. J. Virol. **77:**732–738.
18. **Harbury, P. B., P. S. Kim, and T. Alber.** 1994. Crystal structure of an isoleucine-zipper trimer. Nature **371:**80–83.
19. **Harbury, P. B., T. Zhang, P. S. Kim, and T. Alber.** 1993. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science **262:**1401–1407.
20. **Hartlieb, B., J. Modrof, E. Mühlberger, H. D. Klenk, and S. Becker.** 2003. Oligomerization of Ebola virus VP30 is essential for viral transcription and can be inhibited by a synthetic peptide. J. Biol. Chem. **278:**41830–41836.
21. **Harty, R. N., and P. Palese.** 1995. Measles virus phosphoprotein (P) requires the NH_2- and CO-OH-terminal domains for interactions with the nucleoprotein (N) but only the CO-OH terminus for interactions with itself. J. Gen. Virol. **76:**2863–2867.
22. **Imai, Y., Y. Matsushima, T. Sugimura, and M. Terada.** 1991. A simple and rapid method for generating a deletion by PCR. Nucleic Acids Res. **19:**2785.
23. **Jones, S. M., H. Feldmann, U. Ströher, J. B. Geisbert, L. Fernando, A. Grolla, H. D. Klenk, N. J. Sullivan, V. E. Volchkov, E. A. Fritz, K. M. Daddario, L. E. Hensley, P. B. Jahrling, and T. W. Geisbert.** 2005. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat. Med. **11:**786–790.
24. **Kolesnikova, L., B. Berghöfer, S. Bamberg, and S. Becker.** 2004. Multivesicular bodies as a platform for formation of the Marburg virus envelope. J. Virol. **78:**12277–12287.
25. **Kolesnikova, L., E. Muhlberger, E. Ryabchikova, and S. Becker.** 2000. Ultrastructural organization of recombinant Marburg virus nucleoprotein: comparison with Marburg virus inclusions. J. Virol. **74:**3899–3904.
26. **Lupas, A.** 1996. Coiled coils: new structures and new functions. Trends Biochem. Sci. **21:**375–382.
27. **Lupas, A., M. Van Dyke, and J. Stock.** 1991. Predicting coiled coils from protein sequences. Science **252:**1162–1164.
28. **Martini, G. A., H. G. Kanuff, H. A. Schmidt, G. Mayer, and G. Baltzer.** 1968. Über eine bisher unbekannte, von Affen übertragene Viruserkrankung: Marburg-Virus Krankheit. Dtsch. Med. Wochenschr. **93:**559–571.
29. **Mavrakis, M., L. Kolesnikova, G. B. Schoehn, S., and R. W. H. Ruigrok.** 2002. Morphology of Marburg virus NP-RNA. Virology **296:**300–307.
30. **Modrof, J., C. Möritz, L. Kolesnikova, T. Konakova, B. Hartlieb, A. Randolf, E. Mühlberger, and S. Becker.** 2001. Phosphorylation of Marburg virus VP30 at serines 40 and 42 is critical for its interaction with NP inclusions. Virology **287:**171–182.
31. **Modrof, J., E. Mühlberger, H. D. Klenk, and S. Becker.** 2002. Phosphorylation of VP30 impairs Ebola virus transcription. J. Biol. Chem. **277:**33099–33104.
32. **Mühlberger, E., B. Lötfering, H.-D. Klenk, and S. Becker.** 1998. Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. J. Virol. **72:**8756–8764.
33. **Mühlberger, E., M. Weik, V. E. Volchkov, H.-D. Klenk, and S. Becker.** 1999. Comparison of the transcription and replication strategies of Marburg virus and Ebola virus by using artificial replication systems. J. Virol. **73:**2333–2342.
34. **Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka.** 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J. Virol. **76:**406–410.
35. **Nishio, M., M. Tsurudome, M. Ito, N. Watanabe, M. Kawano, H. Komada, and Y. Ito.** 1997. Human parainfluenza virus type 2 phosphoprotein: mapping of monoclonal antibody epitopes and location of the multimerization domain. J. Gen. Virol. **78:**1303–1308.
36. **Raha, T., E. Samal, A. Majumdar, S. Basak, D. Chattopadhyay, and D. J. Chattopadhyay.** 2000. N-terminal region of P protein of Chandipura virus is responsible for phosphorylation-mediated homodimerization. Protein Eng. **13:**437–444.
37. **Rahaman, A., N. Srinivasan, N. Shamala, and M. S. Shaila.** 2004. Phosphoprotein of the rinderpest virus forms a tetramer through a coiled coil region important for biological function. A structural insight. J. Biol. Chem. **279:**23606–23614.
38. **Raker, V. A., G. Plessel, and R. Lührmann.** 1996. The snRNP core assembly pathway: identification of stable core protein heteromeric complexes and an snRNP subcore particle in vitro. EMBO J. **15:**2256–2269.
39. **Ryan, K. W., and A. Portner.** 1990. Separate domains of Sendai virus P protein are required for binding to viral nucleocapsids. Virology **174:**515–521.
40. **Sanchez, A., M. P. Kiley, H. D. Klenk, and H. Feldmann.** 1992. Sequence analysis of the Marburg virus nucleoprotein gene: comparison to Ebola virus and other non-segmented negative-strand RNA viruses. J. Gen. Virol. **73**(Pt. 2)**:**347–357.
41. **Schneider, U., K. Blechschmidt, M. Schwemmle, and P. Staeheli.** 2004. Overlap of interaction domains indicates a central role of the P protein in assembly and regulation of the Borna disease virus polymerase complex. J. Biol. Chem. **279:**55290–55296.
42. **Schultz, D. E., M. Honda, L. E. Whetter, K. L. McKnight, and S. M. Lemon.** 1996. Mutations within the 5′ nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. J. Virol. **70:**1041–1049.
43. **Shaji, D., and M. S. Shaila.** 1999. Domains of rinderpest virus phosphoprotein involved in interaction with itself and the nucleocapsid protein. Virology **258:**415–424.
44. **Spehner, D., R. Drillien, and P. M. Howley.** 1997. The assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by the phosphoprotein. Virology **232:**260–268.
45. **Tarbouriech, N., J. Curran, C. Ebel, R. W. Ruigrok, and W. P. Burmeister.** 2000. On the domain structure and the polymerization state of the Sendai virus P protein. Virology **266:**99–109.
46. **Tarbouriech, N., J. Curran, R. W. Ruigrok, and W. P. Burmeister.** 2000. Tetrameric coiled coil domain of Sendai virus phosphoprotein. Nat. Struct. Biol. **7:**777–781.